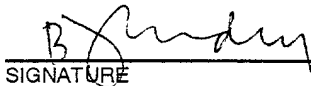


FORM PTO-1390 (REV 11-98)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 1721-21
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) 09/530363 <small>Unassigned</small>
INTERNATIONAL APPLICATION NO. PCT/FR98/02320	INTERNATIONAL FILING DATE 29 October 1998	PRIORITY DATE CLAIMED 30 October 1997
TITLE OF INVENTION METHOD FOR DIAGNOSING IN VITRO PATHOLOGIES ASSOCIATED WITH GENE ARRANGEMENTS AND DIAGNOSIS KITS		
APPLICANT(S) FOR DO/EO/US GABERT		
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:		
1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.		
2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.		
3. <input checked="" type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).		
4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19 th month from the earliest claimed priority date.		
5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)).		
a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau).		
b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau.		
c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).		
6. <input checked="" type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)).		
7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).		
a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau)		
b. <input type="checkbox"/> have been transmitted by the International Bureau.		
c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.		
d. <input type="checkbox"/> have not been made and will not be made		
8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (U.S.C. 371(c)(3))		
9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).		
10. <input checked="" type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).		
Items 11. To 16. Below concern document(s) or information included:		
11. <input type="checkbox"/> An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.		
12. <input type="checkbox"/> An assignment document for recording A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.		
13. <input checked="" type="checkbox"/> A FIRST preliminary amendment.		
<input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.		
14. <input type="checkbox"/> A substitute specification.		
15. <input type="checkbox"/> A change of power of attorney and/or address letter		
16. <input checked="" type="checkbox"/> Other items or information. PTO-1449 and International Search Report, Letter with paper and computer readable copies of Sequence Listing		

09/530363

527 Rec'd PCT/PTO 28 APR 2000

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) Unassigned		INTERNATIONAL APPLICATION NO. PCT/FR98/02320		ATTORNEY'S DOCKET NUMBER 1721-21			
17. <input checked="" type="checkbox"/> The following fees are submitted:				CALCULATIONS PTO USE ONLY			
BASIC NATIONAL FEE (37 C.F.R. 1.492(a)(1)-(5): -- Neither international preliminary examination fee (37 C.F.R. 1.482) nor international search fee (37 C.F.R. 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO.....\$970.00 -- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO.....\$840.00 -- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but international search fee (37 C.F.R. 1.445(a)(2)) paid to USPTO.....\$690.00 -- International preliminary examination fee paid to USPTO (37 C.F.R. 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4).....\$670.00 -- International preliminary examination fee paid to USPTO (37 C.F.R. 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4).....\$96.00 <div style="text-align: right;">ENTER APPROPRIATE BASIC FEE AMOUNT =</div> Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 C.F.R. 1.492(e)).							
						\$ 840.00	
						\$ 130.00	
						\$ 0.00	
						\$ 0.00	
CLAIMS		NUMBER FILED	NUMBER EXTRA	RATE			
Total Claims		15	-20 = 0	X	\$18.00		
Independent Claims		1	-3 = 0	X	\$78.00		
MULTIPLE DEPENDENT CLAIMS(S) (if applicable)				\$260.00			
TOTAL OF ABOVE CALCULATIONS =				\$ 970.00			
Reduction by 1/2 for filing by small entity, if applicable. A Small Entity Statement must also be filed (Note 37 C.F.R. 1.9, 1.27, 1.28).				0.00			
SUBTOTAL =				\$ 970.00			
Processing fee of \$130.00, for furnishing the English Translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 C.F.R. 1.492(f)).				0.00			
TOTAL NATIONAL FEE =				\$ 970.00			
Fee for recording the enclosed assignment (37 C.F.R. 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. 3.28, 3.31). \$40.00 per property				0.00			
Fee for Petition to Revive Unintentionally Abandoned Application (\$1210.00 - Small Entity = \$605.00)				0.00			
TOTAL FEES ENCLOSED =				\$ 970.00			
				Amount to be: refunded \$			
				Charged \$			
a. <input checked="" type="checkbox"/> A check in the amount of \$970.00 to cover the above fees is enclosed. b. <input type="checkbox"/> Please charge my Deposit Account No. 14-1140 in the amount of \$_____ to cover the above fees. A duplicate copy of this form is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-1140. A duplicate copy of this form is enclosed. d. <input type="checkbox"/> The entire content of the foreign application(s), referred to in this application is/are hereby incorporated by reference in this application. NOTE: Where an appropriate time limit under 37 C.F.R. 1.494 or 1.495 has not been met, a petition to revive (37 C.F.R. 1.137(a) or (b)) must be filed and granted to restore the application to pending status. SEND ALL CORRESPONDENCE TO: NIXON & VANDERHYE P.C. 1100 North Glebe Road, 8 th Floor Arlington, Virginia 22201 Telephone: (703) 816-4000							
				 SIGNATURE			
				B.J. Sadoff NAME			
				36,663 April 28, 2000 REGISTRATION NUMBER Date			

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

GABERT

U.S. National Phase of PCT/FR98/02320

Serial No. Unassigned

Filed: April 28, 2000

For: METHOD FOR DIAGNOSING IN VITRO
PATHOLOGIES ASSOCIATED WITH GENE
ARRANGEMENTS AND DIAGNOSIS KITS

Atty. Ref.: 1721-21

Group: Unassigned

Examiner: Unassigned

* * * * *

April 28, 2000

Assistant Commissioner for Patents
Washington, DC 20231

PRELIMINARY AMENDMENT

Sir:

Preliminarily amend the above-identified application as follows.

IN THE SPECIFICATION

Amend the specification as follows:

Insert the attached Sequence Listing for that which is included as pages 25-27 of the
attached application, and renumber subsequent pages as appropriate.

IN THE CLAIMS

Amend the claims as follows:

Claim 3, line 1, delete "or 2".

Claim 5, line 1, delete "or 2".

GABERT

Serial No. Unassigned

U.S. National Phase of PCT/FR98/02320

Claim 6, lines 1 and 2, delete "any one of claims 1 to 5" and insert -- claim 1 --.

Claim 7, lines 1 and 2, delete "any one of claims 1 to 6" and insert -- claim 1 --.

Claim 8, lines 1 and 2, delete "any one of the claims 1 to 7" and insert -- claim 1 --.

Claim 10, line 1, delete "or 9".

Claim 11, lines 1 and 2, delete "any one of the claims 1 to 10," and insert -- claim 1 --.

Claim 12, lines 1 and 2, delete "any one of the claims 1 to 10" and insert -- claim 1 --.

Claim 13, line 2, delete "any one of the claims 1 á 10" and insert -- claim 1 --.

Claim 15, line 1, delete "or 14"

REMARKS

The specification and claims have been amended to place the application in a more traditional format.

The attached paper and computer readable copies of the Sequence Listing are the same.
No new matter has been added.

An early and favorable Action on the merits is requested.

[illegible]

By: B.J. Sadoff
B.J. Sadoff
Reg. No. 36,663

422519

11/PRTS

09/530363
527 Rec'd PCT/PTO 28 APR 2000

1

PATENT APPLICATION

5

TITLE :

Methods for diagnosing in vitro pathologies associated with gene rearrangement and diagnosis kits.

10

The invention relates to the detection of gene rearrangements with exchanges of genetic material. Said rearrangements correspond to the formation of fusion genes through coupling of the translocated part with a genome portion located on the chromosomal partner, or through modification of regulation in a gene expression. As used herein, the word "gene" will mean the gene involved in various rearrangements, while the expression "fusion partners" will make reference to the genome portions coupled with the said gene

20

The invention is more specifically aimed at defining a method and kits for in vitro diagnostic of pathologies associated with such rearrangements.

25

As regards for example leukemias, they are known to be associated with rearrangements of numerous genes, some of which recurrent like the MLL gene.

The MLL gene belongs to the 11q23 chromosomal band of human genome, frequently involved in molecular rearrangements, particularly in acute lymphoblastic leukemia (LAL) and acute myeloid leukemia (LAM) as well.

So far, by cytogenetic assessments it was possible to count about thirty different chromosomal partner bands. Thirteen MLL fusion partners have now been cloned and sequenced, which accounts for 95 % approximately of known rearrangements

In most cases, such rearrangements are associated with grim clinical prognosis , whence the emphasis placed on their evaluation in recent years.

With the development of karyotypes, cytogenetics is one of the methods used conventionally. By this technique it was possible to disclose a large number of rearrangements with a large number of partners in the 11q23 chromosomal band and to assess the prognosis significance of the anomaly. But it comprises numerous false negatives and its success rate does not exceed 50 to 70 % range

The "Southern blot" and *in situ* hybridization are amongst the other known techniques.

The Southern blot technique offers the advantage of highlighting all rearrangements but is hardly usable by clinical laboratories due to its time-consuming, heavy process and because of radioactivity constraints. In fact, therapy decisions demand results within weeks on a case by case basis.

In situ (FISH) hybridization may *a priori* highlight genetic anomalies, however its sensitivity is not always adequate due to the frequent deletions which are often found with translocations, the use of this technique may also yield false negative results.

527 Rec'd PCT/PTO 28 APR 2000

In WO 96/13514, the characterisation and isolation of the TCL-1 gene associated with anomalies is disclosed.

This document aims to characterise all genes of the TCL-1 family and for this, uses a PCR technique that employs degenerated primers.

In the J.Clin, Pathol, 1993, 100, p 527-533, Rotech et al report the interest of anchored PCR to detect a wide spectrum of mature B Cell neoplasma. The described primers are specific to IgH and are not therefore entirely random. In addition, the PCR products obtained are cloned and sequenced, which are very labor intensive characterization techniques.

In Human genetics, 1997, 99, p 237-247, Kehrer - Sawatzki et al analyse the breakpoint regions in the NF1 gene implicated in the type 1 neurofibromatose.

The so-called semi-specific PCR technique employed is performed in such a way as to identify the junction fragment in the breakpoint region, and the sequence being then analysed. This article does not then teach asymmetric amplification to amplify the whole fusion genes and to reveal only genes implicated in the rearrangement.

In PNAS, 1993, 90, p 8538-8542, Corral et al study breaks in the MLL of different translocations and 11q deletions with cloning of certain MLL gene partners for identification, this represents a technique that can be used in research but is not adapted for routine use.

The invention further aims at providing diagnostic kits to implement this method.

The in vitro diagnostic method according to the invention, is characterized in that a patient's DNA is
5 subjected to at least one step of anchored PCR by completing at least one step of asymmetrical amplification, by means of a single pair of primers formed by a specific DNA primer of the gene liable to be involved in a fusion gene, and a complementary random
10 primer, and in that such a gene is only detected in so far as it is involved in the said fusion.

It will be observed that the foregoing arrangements allow one to conveniently amplify any sequence involving the relevant gene, whatever the fusion
15 partner, and even though the length of the sequence associated with the gene be significant. On the contrary, the detection step is specific and does not allow one to detect the gene unless rearranged with a given partner

The primers used in the amplification step are
20 conveniently selected to meet the length, T_m and end-stability criteria, particularly in the case of long fragments' amplification.

Thus, the length of the primers must ensure a stability consistent with elongation. Convenient primers
25 include 25 to 40 nucleotides, and specifically 30 to 35 nucleotides.

The T_m temperature at which half the DNA is in denatured form is advantageously of about 80 to 86°C,

specifically close to 80°C. The sequence base composition is to be selected in such a way as will meet this requirement.

Similarly, one should take into account the stability at the 3' and 5' ends, since the primer end subjected to elongation should be less stable than the opposite end, to avoid the initiation and elongation of non specific PCR products. It is also critical to obviate the formation of duplex and loops on the 3' end which would interfere with the proper primers matching with the DNA or cDNA sequence.

The primers as described above can easily be prepared by means of software products.

The anchored PCR strategy can be conducted on the 3' end as defined hereabove, but on the 5' end as well, in which case an artificial tail is to be added to the gene's 5' end, and can be used as a primer. To that effect the deoxyribonucleotidyltransferase terminal enzyme can be conveniently used.

Rearranged genes can be detected by means of any suitable marker.

As a general rule, specific probes of nucleotide sequences of known fusion partners are put into contact with denatured PCR products, marked for detection in conditions which promote a specific probes-PCR products interaction where a complementarity of bases is present.

PCR products carry a marker (digoxigenine, biotin or fluorophore for example) by which they will be

detected. Such marker is carried by a desoxynucleotide embodied into the PCR products during the second amplification.

5 The probes can be covalently secured on a support, such as 96-well plates

Such covalent bonding can be advantageously obtained through biotinylated probe /plate-streptavidine coupling.

10 Alternatively, this covalent bond can be achieved by means of a probe with phosphorylated end and a carbodiimide residue on the plate or a probe modified by amine residue on one end and bonded by N-oxysuccinimide esters residues.

15 According to a satisfactory method, the ELISA technique is used to specifically detect such nucleotide sequences that include the gene involved in a rearrangement.

20 PCR products in which a marker has been incorporated are allowed to react with an itself marked antibody, directed against the PCR product markers, in conditions promoting an antigen-antibody type reaction, which is detected if present by highlighting the marker of the antibody or of a reaction in which it is involved.

25 According to still another method, the PCR technology which enables PCR products to be detected by hybridization of internal probe and PCR product in solution.

It will be observed that multiple PCR used with different genes marked with different markers from one

another, allow a single test to detect a plurality of rearranged genes involved in a pathology.

A detection alternative, to highlight numerous genes rearrangements on a large number of genes, in a single test, is based on the DNA chips technology and comprises using oligonucleotidic or cDNA probes secured to a miniaturized support. Each probe or hybridization unit may advantageously be individually controlled by an electric field. In another alternative, the internal probes are advantageously immobilized on strips.

The DNA subjected to amplification advantageously corresponds to the cDNA, as obtained by reverse transcription of the RNA extracted from the sample.

Alternatively, the genome DNA extracted from the sample under investigation can be used.

The internal probes are advantageously immobilized on strips.

According to an embodiment of the invention, a reverse transcription step (RT in short) is performed before PCR amplification, to synthesize a cDNA population from the cells' RNA in the sample investigated.

A stable nucleotide sequence at 80 to 90° C T_m is conveniently used to perform this step.

Suitable sequences include a cassette with about 40 to 60 nucleotides with 10 to 20 T-patterns on one end or, alternatively, a random repeated nucleotide pattern.

According to another embodiment of the

invention, the genome DNA or RNA, extracted from the cells of the sample under investigation are subjected to the effect of a compound with the ability to inhibit or to cleave specifically the DNA of the gene under fusion investigation. These are like PNA (polypeptidic nucleic acids) or ribozymes for example. The PCR, or RT-PCR steps as the case may be, are then performed with primers which optionally include cloning sites. The products thus obtained are then set to react with two specific probes of the gene investigated, one located upstream of the break point region ("a" probe) and one downstream ("b" probe) on the one hand, and probes prepared from known partner genes ("c" probes). A positive result on «a» probe and a negative result on «b» probe, leads to conclude to the rearrangement of the gene studied, and a negative result on "c" probes detects the absence of any known fusion product. Where new fusion genes are highlighted by the test, they can be secondarily cloned and sequenced using conventional techniques.

Therefore, this technique contributes valuable information to understand molecular events underlying the cell transformation.

According to an advantageous embodiment of the invention, implemented to detect translocations involving the MLL gene, a cDNA pool is synthesized from the RNA extracted from the sample under investigation with the aid of primers including a cassette of about 30 to 35 nucleotides, complemented by a sequence of 6 or 9 random nucleotide patterns, and an anchored PCR is

performed using a primer located on the MLL' exon 5, as specific sense primer. Where a second amplification cycle is performed, an internal sense primer is used to increase the specificity. The random primer is advantageously
5 selected as complementary to the oligonucleotides cassette used on the reverse transcription step.

The ELISA technique is used to detect fusion transcripts if any, and entails locating equally spaced probes on the fusion partners investigated, in order to
10 encompass all break points.

In an initial step, a specific probe of known MLL' fusion partners is made to contact denatured PCR products marked by digoxigenine in the second amplification cycle, in conditions which promote
15 hybridization where complementarity between bases exists.

In a second step, the resulting products are put into contact with anti-digoxigenine antibodies, coupled with an enzyme with the ability to release a detectable colored product if the antibodies should be
20 secured to PCR products, by reacting with its substrate.

Hybridization between about 37 and 50 °C for 2 to 4 hours, yields satisfactory results.

The probe-PCR products interaction is achieved at a temperature in excess of 30°C, in the 35 to 65°C
25 range, particularly at about 55°C, for 0,5 h to 5 h, and in particular, about 1 h.

Washing conditions are selected in such a way as will produce an optimum signal/noise ratio.

The enzyme substrate is prompted to react with

the probes/ PCR products reagent mixture in the same temperature and time conditions, and the product released if any can be detected, for instance by optical density measurement.

5 The results obtained by this method show that fusion products of the relevant translocations are easily detected through potent signals. Such results are easier to interpret than negative tests.

10 So, some 95 % of MLL gene rearrangements can be detected.

15 In order to detect new MLL-partner gene associations, total RNAs are subjected to the action of MLL gene-specific ribozymes, before the RT-PCR, after which amplification products are made to react with a probe corresponding to the MLL' Exon 5 on the primer 3' end, and then with a still MLL gene-specific second probe, located between the ribozymes' break point and action site, and finally with known partners' probes. A positive signal in the first case and a negative one with
20 the second probe on gene MLL suggests a MLL rearrangement, while a negative signal obtained in the third step means that no known fusion product was detected. Then a new fusion gene can be highlighted.

25 Alternatively, partners can be sought by implementing the above described PCR steps and RT-PCR steps as the case may be, and detecting PCR products by means of DNA chips made up with oligonucleotidic or cDNA probes secured on a miniaturized surface.

 The invention also provides diagnostic kits to

implement the method defined above.

Such kits are characterized in that they include the necessary reagents to perform at least one PCR and detection test and, if required, the reverse transcription and/or reaction with agents such as ribozymes or PNAs with the capability of specifically cutting or inhibiting the gene under rearrangement investigation.

Particularly, such kits include primers for the various reactions and advantageously suitable solvents or buffers, appropriate for carrying out the reactions, particularly for hybridization and washing, and a user's notice.

Preferred kits include fusion partners-specific probes secured on a support. Such probes may for instance be secured on a plate and are such as obtained by coupling a reagent on one of their ends with a plate reagent. These are for instances biotinylated probes on the 5' end secured on streptavidine coating the wells bottom of a micro-plate.

Oligonucleotidic or cDNA probes secured on a miniaturized support (DNA chips) are alternatively used.

According to still another alternative, the internal probes are fixed on strips.

The detection technique can be standardized and alleviated for the detection of fusion genes or fusion transcripts sought, thanks to the possibility of storing such support-plates which carry the probes.

Experiments on cell lines were confirmed in

patients whose gene rearrangement type was already identified, which confirms their clinical value to obtain a molecular diagnostic and to define break points.

5 The value of the method contemplated by the invention is underscored in those cases, particularly AML, where no chromosomal anomaly is evidenced by cytogenetic assessment whereas a molecular rearrangement is detected by molecular biology. The method contemplated by the invention allows one to screen AML patients whose
10 karyotype is not available or was reported as normal and to ascertain whether rearrangements associated with a pathology are present or not.

The invention is therefore particularly valuable for leukemia diagnostic.

15 It is also especially useful in carcinology. In particular, one can mention the solid tumor diagnostic and particularly EWS genes rearrangements in Ewing Tumors. The method contemplated by the invention allows one to detect rearrangements of EWS/FLI1 or of other
20 members of the ETS gene family, such as ERG, ETV1 or E1AF.

As a general rule, the invention provides the necessary tools to obtain a simple, reliable and highly sensitive diagnostic over a large number of samples. The
25 amplification of the initial sample material is also quite valuable since this material was obtained from patients, like blood or bone marrow. A large number of probes can be tested , typically up to about 500 probes on 96-well plates.

An interesting feature of the invention will be found in the arrangements whereby the tests can be automated, especially at the detection step.

Furthermore, as already emphasized, the invention provides means to detect genes which, so far, were not identified as involved in a given disorder.

Other characteristics and advantages inherent in the invention are presented in the following examples, and by referring to figure 1A to 1C which gives the general schedule of the steps used for detecting the fusion transcripts.

Example 1 : Detection protocol of a gene rearrangement with known fusion partners.

(1)The cDNAs are synthesized from the total RNAs in the sample studied, by reverse transcription (RT), then (2) the cDNA pool is amplified by PCR and (3) the transcripts are checked for specificity. Said steps are illustrated by the schedule given on the single figure.

1. RNA Preparation

The cells from the sample studied are placed in a lysate solution with addition of Trizol^R (Life Technologie). Then, chloroform (20% final) is added to the resulting cell lysate, and after 5 min incubation at room temperature, the mixture is centrifugalized 15 min at 4°C and 12,000 g.

Three phases are thus obtained, viz., a colorless aqueous phase containing the RNA, a whitish

intermediate phase containing the DNA, and a red, phenolic organic phase.

Isopropanol is added to the RNA, (500 μ l in 1 ml Trizol^R), then the compound is centrifugated for 10 min at 4°C and 12,000 g, following 10 min incubation at room temperature; the resulting precipitate is then rinsed in 1 ml of 75 % ethanol (5 min at 4°C and 7,500 g).

The precipitate is dried at room temperature before mixing in 10 μ l water and processed with H RNase.

The quantity of RNA extract is calculated by measuring the optical density at 260 nm : concentration (μ g/ μ l) = measured DO x 40 (extinction coefficient) x dilution coefficient x 10⁻³.

2. Reverse Transcription

Superscript^R (Life Technologie, 18064-014) or Expand Reverse Transcrip-tase^R (Boehringer, 1 785 834) are used as enzymatic system, under the following application protocol:

1 μ g RNA is denatured (9,5 μ l volume) 10 min at 70°C, then added to the reactive mixture(10,5 μ l): nucleotides (1 mM) + dTT (10mM) + primer 0,5 μ M) + RNases inhibitors(20 units) + enzyme (50 units of Expand Reverse Transcriptase^R, 200 units of Superscript)^R, all in a buffer suited to either enzymatic system :

Superscript^R : 20 mM Tris HCl, 50 mM KCl, 5 mM MgCl₂

Expand Reverse Transcriptase^R : 50 mM Tris HCl, 40

mM KCl, 5 mM MgCl₂.

The cDNA synthesis is performed through the following cycle : 10 min at 20°C / 45 min at 42°C / 3 min at 99°C.

The samples are then subjected to H.RNase
5 (Boehringer, 786 357 : 2 unites) for 10 min, at 42°C.

The cDNAs are transferred into a final volume of 60 µl (dilution to 1/3) and stored at -20°C.

3 cDNA amplification by PCR

10 The results so obtained are reported with eLONGASE^R (BRL, 10481-018) and Expand Long template PCR System (Boehringer, 175 9060) as enzymatic systems.

Two different amplification programs are used according to the length of the fragments :

15 - amplification of fragments up to 1 kb:

94°C	3 min	
94°C	30 sec	
58°C	30 sec	x 34
72°C	30 sec	
16°C	∞	

20 - amplification of fragments in excess of 1kb

95°C	30 sec
94°C	10 sec / 68°C 8 min x 10
94°C	10 sec / 68°C 8 min + 20 sec per cycle x20
25 68°C	7 min
16°C	∞

In all cases, the reaction takes place in a 50 µl volume adhering to the following conditions: dXTP (500 µM), sens and antisens primers (1 µM), MgCl₂ (3 mM),
30 enzymes (2,5 unites), all in a 50 mM Tris HCl (pH 9,2), 16 mM (NH₄)₂SO₄, 2% DMSO, 0,1% Tween 20 buffer solution.

A second amplification cycle is completed to assess long fragments, using 1 μ l of product obtained from the first PCR. An internal sens primer with respect to the first cycle primer is used with an identical antisens primer ; to perform the ELISA detection , the dTTP is replaced by a dTTP + DIG-dUTP^R mixture(Boehringer ; 1558 706) to the 1 :19 ratio.

4. ELISA detection(Boehringer kit, 1636 111) of probes / PCR products hybrids.

The initial step consists in putting biotinylated specific probes of known partners into contact with PCR products and the hybrids so formed are detected in the next phase.

a. hybridization

With the oligo 5 software, the probes are selected on the sequences of the various partners just upstream of the break points described in each translocation. Thereafter, the probes are biotinylated on the 5' end and purified with HPLC.

- extemporaneous probes fixation on ELISA plates

10 μ l of PCR products are denatured in 10 μ l alkaline solution, then settled in a well with streptavidine coating in the presence of the 7,5 pmol/ml biotinylated probe (220 μ l end volume). The hybridization reaction takes place between 37 and 50°C, for three hours under

stirring.

The anti-DIG anti-body coupled with peroxydase (2 mU in 200 μ l volume) is added after three washings, followed by 30 min incubation at 37°C and by a series of three washings.

1 mg/ml.peroxydase substrate is added (30 min et 37°C). The optical density is then read at 405 nm instead of 492 nm.

- preliminary fixation of biotinylated probes to ELISA plates (R. Giorda et col., 14):

100 μ l (per well) of 0,75 pmol/ μ l probe solution are left 2 hours in incubator at room temperature with stirring.

After washing, 100 μ l of 5x Denhardt's / 0,02% Na azide solution are placed in each well.

The plates can thus be stored at 4°C and used as and when needed : they just need being washed (three times) and then denatured PCR products are to be dropped in 100 μ l hybridization buffer; the rest of the protocol is then completed as described above.

Example 2 : Rearrangements detection protocol for a gene with unknown fusion partners.

Total RNAs extracted from the sample cells are to be first treated by ribosomes as follows : 2 μ g RNA are to be put into contact with ribozymes (1 μ M) in a

buffer solution : MgCl_2 (20 mM) ; Tris HCl pH 8 (50 mM),
to 10 μl volume; the reactive mixture is placed in
incubator for 2 hours at 37°C.

5 The reaction products are collected by precipitation in
absolute alcohol (2,5 volumes), with glycogen and sodium
acetate (0,3 M final) : 30 min in ice followed by 30 min
et 14000 g, 4°C; after rinsing in 75 % alcohol (20 min et
14000 g, 4°C) the precipitates are collected in 10 μl
10 water and subjected to the RT and PCR reactions according
to the invention.

Example 3 : MLL gene rearrangements detection
. MLL translocations

15 The characterization of translocations
involving MLL and fusion partners according to the prior
art is illustrated by the following table.

Table

MLL Fusion Transcripts

Cytogenetic anomaly	Fusion partner	Proteic family	Leukemia type	Reference
t(10;11)(p12;q23)	<i>ABL-1</i>	mABL-intermédiaire I	LAM	Taki T. <i>et al.</i> -1998; (1)
t(1;11)(p32;q23)	AF-1p=eps 15	substrat de EGF Ret	LAM	Bernard O. <i>et al.</i> -1994; (2)
t(1;11)(q21;q23)	AF-1q	cytokine ?	LAM	Tse W. <i>et al.</i> -1995; (3)
t(6;11)(q27;q23)*	AF6	myosine/GLGF	T-ALL ou LAM	Prasad R. <i>et al.</i> -1993; (4)
t(11;19)(q23;p13)*	EEN	domaine SH3	LAM	So C. <i>et al.</i> -1997; (5)
t(5;11)(q31;q23)	AF-5	domaine GAP + NSL	CMML enfants	Berkhardt A. -1997r; (6)
t(6;11)*(q21;q23)	AF6 q21	forkhead	MDS/LAM	Bernard O. <i>et al.</i> -1997; (7)
t(X;11)(q13;q23)	AFX1	forkhead	LAM	Corral J. <i>et al.</i> -1993; (8)
t(4;11)(q21;q23)*	AF4	riche en ser., pro.	ALL	Gu Y. <i>et al.</i> -1992; (9)
t(9;11)(p22;q23)	AF9	riche en ser., pro.	LAM	Nakamura T. <i>et al.</i> -1993; (10)
t(11;19)(q23;p13)	ENL	riche en ser., pro.	ALL	Tkachuk D. <i>et al.</i> -1992; (11)
t(10;11)(p12;q23)	AF10	doigt de zinc + LZ	LAM	Chaplin T. <i>et al.</i> -1995; (12)
t(11;17)(q23;q21)	AF17	doigt de zinc + LZ	LAM	Prasad R. <i>et al.</i> -1994; (13)
t(11;16)(q23;p13)	CBP	adaptateur transcriptionnel	LAM secondaire	Taki T. <i>et al.</i> -1997; (14)
t(11;22)(q23;q13)	p300	adaptateur transcriptionnel	LAM secondaire	Ida K. <i>et al.</i> -1997; (15)
t(11;19)(q23;p13.1)	ELL	fact. d'élongation ARN pol. II	LAM	Thirman M. <i>et al.</i> - 1994; (16)
t(11;22)(q23;q11,2)	hCDCrel	cycle de division cellulaire	LAM	Megonigal M. <i>et al.</i> -1998; (17)
(+11)*	MLL	antagoniste de polycomb	LAM	Bernard O. <i>et al.</i> - 1995; (18)
Aucune	MLL	antagoniste de polycomb	LAM	Schichman S. <i>et al.</i> -1994; (19)
t(11;15)(q23;q15)	AF-15	aucune homologie	T-ALL	Kuefer M. <i>et al.</i> -1997r; (20)

* = the fusion transcript may be present with a normal caryotype

r = congress' abstract

The research completed on MLL and its partners has highlighted the following facts :

1. Only the chimerical protein obtained by fusion of the NH₂ part of MLL with the C terminal end of the partner seems to be part of the tumorigenic factors

2. In spite of the heterogeneousness of MLL break points, they are all distributed between the gene's exon 5 and exon 11; due to the MLL-specific patterns conservation, the NH₂ parts in fusion proteins are homologous.

3. A very large number of partners exist : at least as many as in the ALL's Igs or TCRs, where known partners account for more than 95 % of MLL translocations described so far. Such partners do not exhibit any actual structural homology ; AF9, AF4 and ENL only are homologous while AF10 and AF17 seems to belong to a new genes family. MLL may be self-associated through a duplication phenomenon

. AML cells assessment

The following results were obtained with leukemia cell lines (human AML) which exhibited particularly 11q23/6q27 rearrangements through cytogenetic assessment. The cell line can thus be used as positive check for the t translocation (6 ; 11).

The relevant cells are part of the ML-2 line (DSM ACC15) cultivated in a RPMI (90%) + SVF 510%) medium. After completion of the culture process, the

cells were frozen in DMSO (5.10^6 /ml) for storage or lysed in (Trizol^R) solution to extract nucleic acids.

The TF1 cell line (sampled from a patient with erythroleukoblastosis without 11q23 anomaly) was used for control purpose at various stages in the process (T. Kitamura et al, 21.).

RNAs are to be extracted from the cells as indicated above and subjected to RNase H.

. reverse transcription

The process is the same as described in the example 1 using a 5' sequence, 9 random repeat patterns primer

CGTCGTCGTG AATTCCTAGA TCTTCTAGAT ATGTTNNNNN NNNN

(SEQ ID N° 2, 44 nucleotides, $T_m = 84^\circ\text{C}$).

. amplification

Amplification is obtained as described in the example 1 using the following sens primers :

- on the first cycle, a sequence primer

AGCCCAAGTT TGGTGGTCGC AATATAAAGA AG

(SEQ ID N° 3, 32 nucleotides, $T_m = 84^\circ\text{C}$), and

- on the second cycle an internal sequence primer

GCCGAATTCA TGCCTTCCAA AGCCTACCT

(SEQ ID N° 4, 29 nucleotides, $T_m = 86^\circ\text{C}$), and

a sequence primer used as random primer

CGTCGTCGTG AATTCCTAGA TCTTCTAGAT ATGTT

(SEQ ID N° 5, 35 nucleotides, Tm = 81°C).

5 . detection

- hybridization

A specific probe for each MLL partner was defined before the break point of the relevant positive control. The signal/noise ratio obtained by ELISA test reflects the
10 detection efficiency OF each probe. For ENL and duplication, the first value of the ratio corresponds to washing in a full solution, whereas the second value was obtained with ½ diluted solution.

The biotinylated probes used are characterized by Tm
15 comprised between 71°C and 75°C, calculated according to the method of the richness in GC by means of a software.

- optical density measurement

The ODs are to be measured for each fusion
20 transcription. The results obtained show that the various partners are detected. Strong signals allow one to easily interpret the results against negative controls.

Moreover, the use of probes defined downstream the break points described in the literature, allows one
25 to locate the molecular event on the relevant gene.

- new partners detection

The RNAs from t(9;11)positive control on Monomac 6) cell line and TF1 line are to be subjected to ribozymes' action.

30 Two ribozymes with specific enzymatic action of

unaltered MLL gene are used, their cleaving sites are located downstream of the break points region. Such ribozymes respective sequences are as follows :

5 - ribozyme 1 : CUCCAGCUGA UGAGUCCGUG AGGACGAAAC CUUUGG
(SEQ ID N° 6)

- ribozyme 2 : CUGGAAUCUG AUGAGUCCGU GAGGACGAAA UUUUCUUC
(SEQ ID N° 7).

10 Underscored sequences correspond to MLL complimentary sequences about the cleavage points and the plain sequences mean the unpaired sequences leading to the formation of secondary structures required by the ribozymes' catalyst action. Cleaving occurs on the 3'
15 end of the nucleotide following the uracil make up to adenine .

The reaction products were converted into cDNA, and then subjected to amplification by means of a pair of primers located on either side of the cutting points.

20 Therefore, the invention provides the molecular tools and a diagnostic method which can be used on a large number of patients to identify the various MLL partners and relevant break points, and to better understand the development mechanisms of pathologies
25 associated with genes rearrangement, e.g. : leukemiagenesis mechanisms underlying the MLL gene rearrangement.

BIBLIOGRAPHIE

1. Taki T. et al, Blood 1998, Aug 15 ; 92(4) : 1125-30
Bernard O et al 1997
- 2 Bernard O.A. et al, Oncogene 1994 ; 9:1039-1045.
3. Tse W. et al, Blood 1995 ; 85:650-656.
4. Prasad R. et al, Cancer research 1993; 53; 5624-5628.
5. So C.W. et al, USA 1997; P.N.A.S. 94: 2563-2568.
6. Borkhardt A. et al, Abstract 2170, december 8, 1997. Poster Session : Tumor Suppressor genes and anti-oncogènes.
7. Bernard O.A. et al, Gene Chromosomes Cancer, 1998, jul. 22(3) : 221-4
8. Corral J. et al, P.N.A.S., USA, 1993;90:8538-8542.
9. Gu Y. et al, Cell 1992; 71:701-708.
10. Nakamura T. et al, P.N.A.S. USA, 1993; 90 4631-4635.
11. Tkachuk D.C. et al, Cell 1992 ; 71:691-700.
12. Chaplin T. et al, Blood 1995 ; 85 : 1435-1441.
13. Prasad R. et al, P.N.A.S. USA, 1994; 91: 8107-8111.
14. Taki T. et al, 1997, Blood 1997, vol. 89, n° 11, pp 3945-3950.
15. Ida K. Blood 1997, Dec 15, 90(12) : 4699-704
16. Thirman M.J. et al, P.N.A.S. USA, 1994 ; 91:12110-12114.
17. Megonigal M.D. et al, P.N.A.S., USA, 1998 ; 95 : 6413-6418.
18. Bernard O.A. et al , Leukemia, 1995, vol.9:1487-1490,
19. Schichman S. et al, 1994, Cancer Research, vol.54, 4277-4280.
20. Kuefer M. et al, Abstract 1419, . december 8, 1997, Poster Session : Chromosome Reamangements
21. Kitamura T. et al, J Cell Physiol 1988 ; 140:323-334.

SEQUENCES LIST

5 (1) GENERAL:

(i) APPLICANT:

- (A) NAME Jean GABERT
(B) ADDRESS: 70 Chemin du Lancier
10 (C) CITY: MARSEILLE
(E) CONTRY: FRANCE
(F) POSTAL CODE : 13008

(ii) TITLE OF THE INVENTION: In vitro
15 diagnostic method and kits in disorders associated with
gene rearrangement.

(iii) NUMBER OF SEQUENCES: 7

20 (iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
(B) HARDWARE: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

25 (OEB)

(2) INFORMATION FOR SEQ ID NO: 1:

30 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 52 pairs of bases
(B) TYPE: nucleotide
(C) NUMBER OF STRANDS: single
(D) CONFIGURATION: linear

35 (ii) TYPE OF MOLECULE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

40 CGTCGTCGTG AATTCCTAGA TCTTCTAGAT ATGTTTTTTT TTTTTTTTTT VV 52

(3) INFORMATIONS ON SEQ ID NO: 2:

45 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 pairs of bases
(B) TYPE: nucleotide
(C) STRANDS NUMBER: single
50 (D) CONFIGURATION: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

5 CGTCGTCGTG AATTCCTAGA TCTTCTAGAT ATGTTNNNNN NNNN 44

(4) INFORMATION ON SEQ ID NO: 3:

- 10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 pairs of bases
(B) TYPE: nucleotide
(C) STRAND NUMBER: single
15 (D) CONFIGURATION: linear

(ii) MOLECULE TYPE: DNA

20 (xi) SEQUENCE DESCRIPTION : SEQ ID NO: 3:

AGCCCAAGTT TGGTGGTCGC AATATAAGA AG 32

25 (5) INFORMATION ON SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 pairs of bases
(B) TYPE: nucleotide
(C) STRAND NUMBER: single
30 (D) CONFIGURATION: linear

(ii) MOLECULE TYPE: DNA

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GCCGAATTCA TGCCTTCCAA AGCCTACCT 29

40 (6) INFORMATION ON SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 pairs of bases
(B) TYPE: nucleotide
(C) STRAND NUMBER: single
45 (D) CONFIGURATION: linear

50 (ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CGTCGTCGTG AATTCCTAGA TCTTCTAGAT ATGTT

35

(7) INFORMATIONS ON SEQ ID NO: 6:

5

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 36 pairs of bases
 - (B) TYPE: nucleotide
 - (C) STRAND NUMBER: single
 - (D) CONFIGURATION: trefoil

10

(ii) MOLECULE TYPE: DNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CUCCAGCUGA UGAGUCCGUG AGGACGAAAC CUUUGG

36

20

(8) INFORMATIONS OB SEQ ID NO: 7:

25

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 38 pairs of bases
 - (B) TYPE: nucleotide
 - (C) STRAND NUMBER: single
 - (D) CONFIGURATION : trefoil

30

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

35

CUGGAAUCUG AUGAGUCCGU GAGGACGAAA UUUUCUUC

38

CLAIMS

1/ An in vitro diagnostic method of pathologies associated with gene rearrangement, wherein a patient's DNA or cDNA is subjected to a step of anchored PCR, characterized in that it comprises, in combination,

- one or several steps of asymmetrical PCR, carried out in a non specific manner, of gene rearrangements, by using a single pair of primers consisting in one primer specific of the nucleotidic sequence corresponding to gene liable to be involved in a fusion gene and one random primer, and

- a detection step of the PCR products, carried out by means of markers specific of gene rearrangements, in order to only detect the genes involved in a rearrangement, in their whole.

2/ The method according to claim 1, characterized in that the primers used in the amplification step include 25 to 40 nucleotides and T_m is of 75 to 85°C.

3/ The method according to claim 1 or 2, characterized in that the PCR products are marked in view of the detection step and are denatured, then put into contact with nucleotidic sequences specific of the nucleotidic sequences of the fusion partners.

4/ The method according to claim 3, characterized in that the probes are covalently secured on a support.

5/ The method according to claim 1 or 2,

characterized in that the PCR products are denaturated and contacted with probes marked in solution.

5 6/ The method according to any one of claims 1 to 5, characterized in that to obtain cDNA, sequences including a cassette of 40 to 60 nucleotides and 10 to 20 T patterns on one end or a random repeat of nucleotide pattern, with T_m of 80 to 90°C are used as primers.

10 7/ The method according to any one of claims 1 to 6, characterized in that it comprises the following steps :

- the genome DNA, or RNA, extracted from the sample cells under investigation, are subjected to the action of a compound capable of cleaving or inhibiting specifically the DNA or RNA of the gene ,the fusion of which is under investigation
- 15 - the PCR steps, are performed and the products so obtained are allowed to react first with two specific probes of the gene under investigation one upstream and one downstream and next, using probes prepared from known partner genes,
- 20

where a positive detection on the upstream probe and a negative detection on downstream probe in the first case, evidencing a rearrangement of the relevant gene, and a negative detection in the second case

25 evidencing that no known fusion product was detected or, alternatively,

- the PCR products are allowed to react with a plurality of probes secured on a miniaturized support, the probe PCR products, specific hybridization highlighting the

partner of the fusion gene.

8/ The method according to any one of the claims 1 to 7, characterized in that it is applied to the detection of translocations involving the MLL gene, and includes

- the RT synthesis of a cDNA pool from the RNA extracted from the sample under investigation, using primers which include a cassette of 30 to 35 nucleotides complemented by a sequence of 6 or 9 random nucleotide patterns,
- the performance of an anchored PCR is completed using a primer located on the MLL' exon 5 as specific sense primer, optionally followed by a second amplification cycle, using an internal sense primer with respect to the first one, where the random primer is the same on each cycle and complementary to the oligonucleotide cassette used in the RT step.

9/ A method according to claim 8, characterized in that the detection of fusion transcripts, if any, includes putting :

- a specific probes of known MLL fusion partners, into contact with denatured PCR products marked by digoxigenine during amplification, in such conditions as will allow hybridization to occur where complimentary bases are present,
- resulting products into contact with anti-digoxigenine antibodies, such antibodies being coupled with an enzyme, capable of reacting with its substrate by releasing a colored product which can be detected if the antibodies should be bonded to PCR products, and then

APR 1990

- the probe/PCR product reactive mixture into contact with the enzyme substrate, and the product so formed if any, can be detected.

5 10/ A method according to claim 8 or 9, characterized in that to detect new MLL-partner gene associations, total RNAs are subjected to the action of ribozymes MLL- gene specific before RT-PCR, then the amplification products are allowed to react first with a probe corresponding to MLL exon 5 sequence on the 3' end
10 of the primer used, then with a second MLL gene-specific probe located between the break points and ribozymes action site, and finally with known partner probes.

11/ Application of the method according to any one of the claims 1 to 10, to leukemia diagnostic.

15 12/ Application of the method according to any one of the claims 1 to 10, to solid tumor diagnostic, such as Ewing tumors.

13/ Diagnostic kits to implement the method according to any one of the claims 1 à 10, characterized
20 in that such kits include the necessary reagents to perform the PCR and detection test, and as the case may be, the reverse transcription and/or reaction with agents capable of inhibiting or cleaving the gene, such kits further including primers for the foregoing various
25 reactions and, preferably, the suitable solvents or buffers.

14/ Kits according to claim 13, characterized in that they comprise agents capable of cleaving or blocking the gene of the polypeptidic nucleic acids or of

the ribozymes.

15/ Kits according to claim 13 or 14,
characterized in that they include oligonucleotide probes
to perform the hybridations at the detection step, such
5 probes being secured on a medium such as a multiwell
plate, such as obtained by coupling a reagent on one of
their ends with a reagent on the plate, for example by
coupling biotin on their 5' end on streptavidine covering
the bottom of a micro plate wells, or under still another
10 embodiment, such oligonucleotide probes are secured on a
miniature medium.

4475 2-3-1100000

Figure 1A

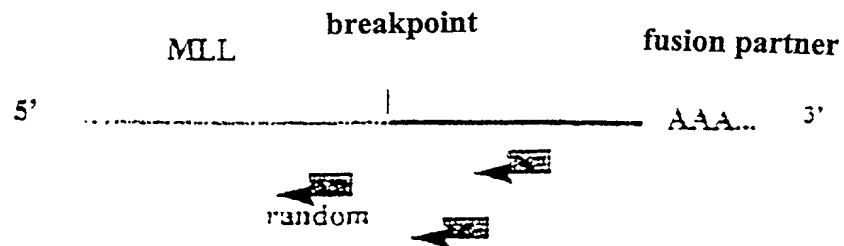
Reverse transcription

Figure 1B

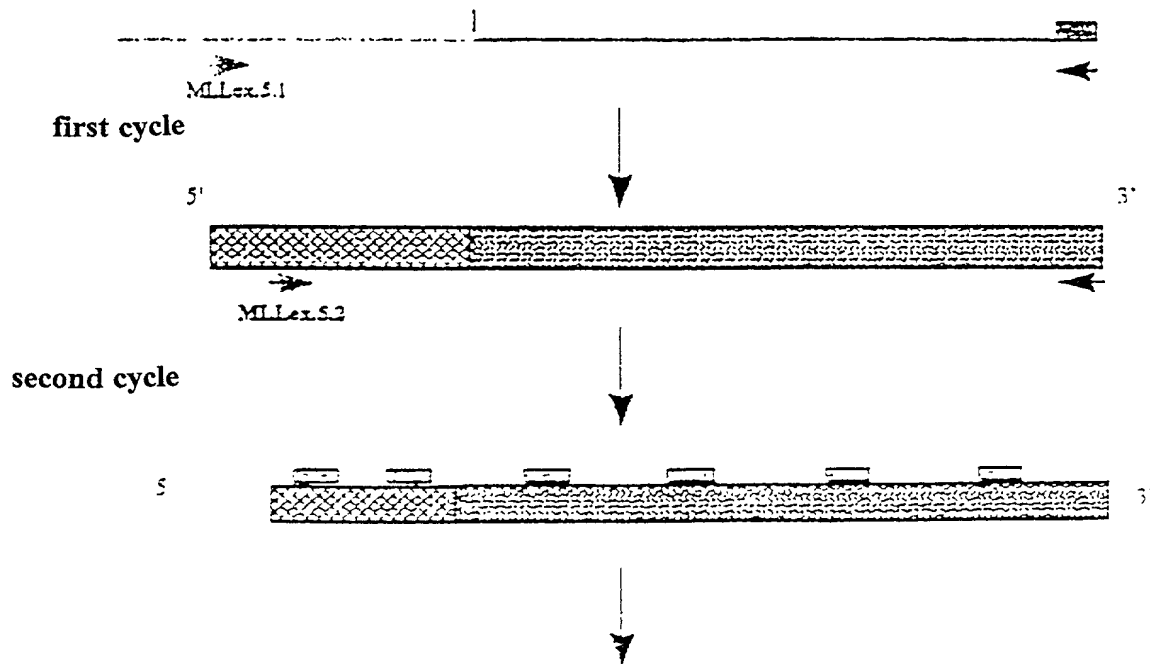
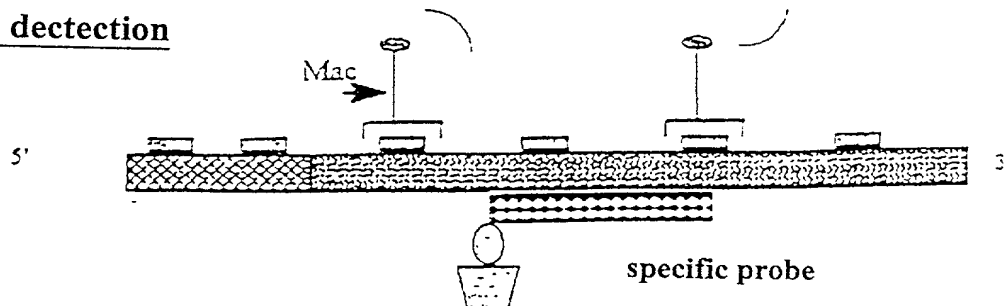
Long distance PCR

Figure 1C

ELISA detection

RULE 63 (37 C.F.R. 1.63)
DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I believe I am the original first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: Method for diagnosing in vitro pathologies associated with gene arrangements and diagnosis kits

the specification of which (check applicable box(s)):

- ☐ is attached hereto.
☐ was filed on _____ as U. S. Application Serial No. _____
☐ was filed as PCT international application No. PCT/FR/98/02320 on October 29, 1998
 and (if applicable to U.S. or PCT application) was amended on November 9, 1999

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with 37 C.F.R. 1.56. I hereby claim foreign priority benefits under 35 U.S.C. 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed or, if no priority is claimed, before the filing date of this application:

Prior Foreign Application(s):

Application Number	Country	Day/Month/Year Filed
97 13656	FR	30/10/1997

I hereby claim the benefit under 35 U.S.C. 120/365 of all prior United States and PCT international applications listed above or below and, insofar as the subject matter of each of the claims of this application is not disclosed in such prior applications in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose material information as defined in 37 C.F.R. 1.56 which occurred between the filing date of the prior applications and the national or PCT international filing date of this application:

Prior U.S./PCT Application(s):

Application Serial No.	Day/Month/Year Filed	Status: patented, pending, abandoned
PCT/FR 98/02320	29/10/1998	Pending

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon. And I hereby appoint NIXON & VANDERHYE P.C., 1100 North Glebe Rd., 8th Floor, Arlington, VA 22204-4714, telephone number (703) 816-4000 (to whom all communications are to be directed), and the following attorneys thereof (of the same address) individually and collectively my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent: Arthur R. Crawford, 25327; Larry S. Nixon, 25640; Robert A. Vanderhye, 27076; James T. Hosmer, 30184; Robert W. Faris, 31352; Richard G. Besha, 22770; Mark E. Nusbaum, 32348; Michael J. Keenan, 32106; Bryan H. Davidson, 30251; Stanley C. Spooner, 27393; Leonard C. Mitchard, 29009; Duane M. Byers, 33363; Paul J. Henon, 33626; Jeffry H. Nelson, 30481; John R. Lastova, 33149; H. Warren Burnam, Jr., 29366; Thomas E. Byrne, 32205; Mary J. Wilson, 32955; J. Scott Davidson, 33489; Jerry D. Craig, 38026.

(1) Inventor's Signature

100 Inventor's Name (typed) Jean GABERT FR
 First Middle Initial Family Name Citizenship

Residence (City) MARSEILLE FRX (State/Foreign Country) FRANCE

Post Office Address Les Jardins de Marseillevoyre 70 chemin du Lancier Zip Code 13008

(2) Inventor's Signature

Inventor's Name (typed) _____
 First Middle Initial Family Name Citizenship

Residence (City) _____ (State/Foreign Country) _____

Post Office Address _____ Zip Code _____

(3) Inventor's Signature

Inventor's Name (typed) _____
 First Middle Initial Family Name Citizenship

Residence (City) _____ (State/Foreign Country) _____

Post Office Address _____ Zip Code _____

FOR ADDITIONAL INVENTORS, check box ☐ and attach sheet with same information and signature and date for each.